Pakistan Journal of Biological Sciences 5 (1): 43-46, 2002
© Asian Network for Scientific Information 2002

Micropropagation of *Annona squamosa* L. Using Nodal Explants

S.A. Farooq, T.T. Farooq and [1]T.V. Rao
Department of Biology, College of Science,
Sultan Qaboos University, P.O. Box-36, PC 123, Sultanate of Oman
[1]Tissue Culture Laboratory, Department of Botany, Osmania University, Hyderabad 500007

Abstract: Large-scale micropropagation has been successfully achieved in *Annona squamosa* L. from nodal explants. Several media with different hormones and culture conditions were tried in standardization of *in vitro* propagation of this plant. Effect of various surface sterilants in eliminating the contamination from the explants collected from the field has been worked out. Activated charcoal has a profound effect in preventing the polyphenol leaching in cultures. BAP, Kinetin, IAA, IBA and NAA were used either alone or in combination to induce shoot buds. Explants produced shoot buds in 7-14 days. Effect of hormones on rhizogenesis was studied. IBA was found to be more effective in root induction. True to type plantlets were obtained from the explants from elite trees. Present protocol is rapid, reproducible and less cumbersome over the conventional methods.

Key words: Micropropagation, *in vitro*, *Annona squamosa*, nodal explants, microbial contamination, rhizogenesis

Introduction

Advances in plant biotechnology research in the last decade has opened new vistas in the propagation of plants with improved resistance to diseases, pests, herbicides and stress etc. Micropropagation of fruit trees is an invaluable aid in the production of elite, disease free, breeding true-to-type in large numbers in short duration.

Annona squamosa L. (Custard apple) is a favourite table fruit of common man in Indian subcontinent. It belongs to family Annonaceae. It is cultivated throughout the tropical and subtropical regions of the world for its delicious and nutritive fruits. The pulp surrounding the seeds of ripe fruit is very delicious and nutritionally valuable with over 20% sugars. The fruit is usually eaten fresh, besides making an ingredient of refreshing drinks and also used to flavour the icecreams and puddings. A highly purgative tea is made from the roots and crushed leaves. Seeds have insecticidal properties and have oil that can be used in paint industry, while the seed cake is used as a manure.

The clonal propagation in *Annona* species is carried out by grafting and budding which are time consuming, while seedling rootstocks are highly variable resulting in the decreased productivity (George and Nissen, 1987). The seed propagation results immense variability affecting yield, size and quality of fruits. Due to the lack of germplasm of improved varieties its cultivation is limited. Micropropagation has been used in other species of *Annona* viz. Cherimoya *A. cheimola* (Tizzari *et al.*, 1990; Rasai *et al.*, 1995) and *A. muricata* (Lemos and Blake, 1996), but the reports on *Annona squamosa* are scanty. Present study deals with the establishment of standard protocols for the micropropagation of the large-scale rapid multiplication of improved genotypes, free of seed borne bacterial and viral diseases.

Materials and Methods

Plant material of *Annona squamosa* var. Balanagar was obtained from Fruit Research Station, Medak, A.P. in 1998. Nodal explants of 3 to 4 cm in length were collected from the upper, middle and lower regions of the newly sprouted branches of mature trees. Leaves were trimmed and explants were washed with enzyme based detergent for 20 min, followed by rinsing in chloroxylenol and washing several times with sterile distilled water containing 0.1% Tween. Damaged cut ends were removed, trimmed to a size of 1 to 1.5 cm with each explant having at least one node. To eliminate the contamination in the cultures, a comparative study was undertaken with various sterilants (Table 1) viz., Mercuric chloride, Sodium hypochlorite, Hydrogen peroxide, Silver nitrate and antibiotics such as Tetracycline, Rifampicin, Polymyxin B and Bavistin (systemic fungicide) and their efficiency was evaluated.

Selection of an appropriate culture medium and the use of correct growth regulators were critical for the optimum growth response of the explants. Three different types of media such as MS medium (Murashige and Skoog, 1962) WPM (Woody Plant Medium, Lloyd and McCrown, 1980) and SH medium (Schenk and Hilderbrandt, 1972) along with different growth regulators were used.

To prevent the leaching of polyphenols from the cut ends of the explants, the following procedures were employed and their effectiveness was studied. I) Pretreatment of explants before inoculation with antioxidant solution (Citric acid 150 mg/l + Ascorbic acid 100 mg/l). ii) Adding antioxidants such as Charcoal (0.1 to 0.5 %), Polyvinylpyrrolidone PVP (200 to 700 mg/l), Ascorbic acid (100 mg/l), Citric acid (150 mg/l) to the medium. iii) Incubation of explants in darkness prior to inoculation. All the aseptic manipulations were carried out in laminarflow. After inoculation, cultures were incubated in cool white fluorescent light (2000 lux) at photoperiods ranging from 12 to 18 hours at a temperature of 24 ± 2 °C. Experiments were conducted with different light intensity viz. 1000-4000 lux. The explants were oriented in horizontal, vertical and slant positions to find out optimum response. The response of the explants in the culture was estimated as the ratio of number of explants responding to the treatment to the total number of the explants inoculated, and is expressed as percentage.

Results and Discussion

The success of micropropagation of *Annona squamosa* through nodal culture involves a number of factors, which affect directly or indirectly the proper establishment of explants in the medium. These are systematically studied to standardize a suitable protocol for the large-scale *in vitro* clonal propagation.

Of all the treatments for controlling contamination, the most efficient response (85.33%) was obtained by immersion and continuous agitation of explants in 0.1% Mercuric chloride for 15 min. followed by rinsing twice with sterile distilled water. Longer treatments were found to cause irreversible damage to

Table 1: Response of explants to the sterilizing agents

Sterilizing agents	Concentration (% or mg/l)	Duration (min)	% Response ± SD	Degree of toxicity
Sodium hypochlorite	2.0	5	24.33 ± 0.47	0
		10	25.02 ± 0.85	0
		15	26.00 ± 1.73	0
		20	26.36 ± 1.23	+
Hydrogen peroxide	12.0	5	25.66 ± 1.65	0
		10	48.60 ± 0.98	0
		15	50.66 ± 0.93	+
		20	50.88 ± 0.98	++
Silver nitrate	1.0	5	24.33 ± 0.46	0
		10	25.02 ± 0.84	0
		15	48.60 ± 0.98	+
		20	50.05 ± 1.70	++
Mercuric chloride	0.1	5	25.66 ± 1.69	0
		10	49.33 ± 0.93	0
		15	85.33 ± 0.47	0
		20	83.70 ± 0.70	++
Antibiotics:				
Tetracycline	50		9.57 ± 1.65	0
	100		24.33 ± 0.46	+
	200		47.94 ± 0.08	++
Rifampicin	50		25.02 ± 0.84	0
	100		50.66 ± 0.97	0
	200		52.05 ± 0.08	++
Polymyxin	50		12.16 ± 0.22	0
	100		24.66 ± 0.46	+
	200		49.33 ± 0.93	++
Bavistin	50		8.37 ± 0.14	0
	100		24.66 ± 0.46	0
	200		34.22 ± 0.85	++

0 = No toxicity, + = less than 50%, ++ = more than 50%

Table 2: Effect of antioxidants in controlling the polyphenol leaching into the media

Antioxidant	Conc. (mg/l)	% Response ± S.D
Polyvinyl pyrrolidone	200	0
	400	0
	700	51.32 ± 0.93
Ascorbic acid	100	26.00 ± 1.41
Citric acid	150	26.00 ± 1.50
Citric acid + Ascorbic acid	100 + 150	51.33 ± 0.93
Activated Charcoal	50	51.33 ± 0.98
	100	51.39 ± 0.93
	200	60.02 ± 1.18
	400	75.66 ± 0.47
Activated Charcoal + dark	100	91.88 ± 0.15

the explants, while treatment of less than 15 min. were relatively less efficient (49.33%) (Table 1). Treatment of explants with 12% Hydrogen peroxide or 1% Silver nitrate over 15 minutes resulted in 50% surface sterilization but most of them turned black due to toxicity. Sodium hypochlorite was found to be least effective in controlling the contamination.

Different antibiotics and fungicides at varying concentrations were incorporated into the medium to eliminate the microbial contaminants from the explants (Table 1). Most of these produced undesirable effects on explants such as delay in bud induction and fewer buds per explant. Philips *et al.* (1981) reported similar observations in *Helianthus tuberosus*. Of all the antibiotic treatments, Rifampicin (100 mg/l) was found to be very effective in controlling the bacterial contamination. Polymyxin B and Tetracycline (100 mg/l) were found to be ineffective and toxic to the explants. Bavistin (100 mg/l) was found to be least toxic and was effective in controlling only the fungal contamination. These results are in conformity with the reports of D'Silva and D'Souza (1993) in cashew nut. The use of antibiotics to control the growth of contaminants in the medium was earlier reported by Pollock *et al.* (1983) and fungicides by Shields *et al.* (1984).

The nodal explants of *Annona squamosa* release a number of polyphenol compounds into the medium, which hamper its establishment. To prevent the polyphenol leaching, various antioxidants were incorporated in the medium. The best response (91.88%) was obtained with activated Charcoal and dark incubation of the cultures (Table 2). Explants showed faster shoot bud induction along with the production of maximum number of buds. At lower concentrations of Charcoal lesser response was obtained. The advantage of Charcoal in the medium was earlier reported by Fridburg *et al.* (1978), Weatherhead *et al.* (1978), D'Silva and D'Souza (1993). Activated charcoal absorbs the polyphenolic compounds and the dark incubation minimizes the synthesis of polyphenols by explants. Addition of Citric acid and Ascorbic acid (100 mg/l) individually to the medium was not effective in preventing the leaching and resulted in the discoloration of the explants. Pre-treatment of explants with Citric acid and Ascorbic acid (150 + 100 mg/l) was partially effective with only 51.33% of explants responding. PVP at lower concentrations (100 to 400 mg/l) was not effective in controlling the leaching of polyphenols, whereas, higher concentrations (500 to 800 mg/l) delayed the shoot bud induction.

Slightly horizontal orientation of the explants in culture medium produced the best response. In the vertical orientation the response was relatively less. Explants placed in the inverted position resulted in delayed shoot bud induction and callus production was prominently observed at the cut ends. The present findings are not in agreement with the observations of Amin and Jaiswal (1987) in guava, where initial culturing of explants in the vertical plane and then shifting to horizontal position resulted in maximum shoot induction. In apple, inverted orientation of explants gave maximum shoot bud induction (Zimmerman, 1989).

Table 3: Effect of hormones on the shoot bud induction

Hormone	Concentration (mg/l)	% Response ± S.D.	No. of shoot buds	No. of days
Kinetin (KN)	0	0	0	7 to 14
	0.2	25.75 ± 1.06	1	7 to 14
	0.4	27.06 ± 0.50	1	7 to 14
	0.6	28.18 ± 1.28	1	5 to 7
	0.8	50.00 ± 0.20	1	5 to 7
	1	51.58 ± 1.12	1	5 to 7
	2	52.09 ± 1.48	2	4 to 8
	4	49.12 ± 1.24	1	
Benzyl Amino	0	0	0	7 to 14
Purine (BAP)	0.2	25.43 ± 0.61	1	7 to 14
	0.4	16.19 ± 1.68	1	6 to 10
	0.6	50.79 ± 1.11	1	8 to 10
	0.8	74.73 ± 1.26	2	4 to 6
	1	75.49 ± 0.69	2	4 to 6
	2	76.22 ± 1.13	2	4 to 6
	4	74.95 ± 1.02	1	
Kinetin + Benzyl	0 + 0	0	0	
Amino Purine	0.2 + 0.2	50.66 ± 0.93	1	7 to 14
	0.4 + 0.4	76.20 ± 1.65	1	7 to 14
	0.6 + 0.6	96.10 ± 0.06	3 to 4	5 to 7
	0.8 + 0.8	73.35 ± 1.23	1 to 2	5 to 7
	1.0 + 1.0	74.80 ± 1.78	1 to 2	7 to 14
	2.0 + 2.0	72.87 ± 0.78	1	7 to 14
	4.0 + 4.0	70.87 ± 1.02	1	7 to 14
Kinetin + Benzyl Amino	0.6 + 0.6 + 0.0	96.10 ± 0.06	3 to 4	7 to 14
Purine + Indole Acetic Acid	0.6 + 0.6 + 0.2	74.83 ± 2.14	1 to 2	7 to 14
	0.6 + 0.6 + 0.4	73.44 ± 1.22	1	7 to 14
	0.6 + 0.6 + 0.6	50.25 ± 2.09	1	7 to 14
	0.6 + 0.6 + 0.8	49.91 ± 2.05	1	7 to 14
Indole Acetic Acid (IAA)	0 to 100	0		
Naphthalene Acetic Acid (IAA)	0 to 100	0		
Indole-3-Butyric Acid (IBA)	0 to 50	0		
	55	20.47 ± 0.66		
	70	27.29 ± 1.00		
	100	40.95 ± 1.34		
	110	34.12 ± 3.76		

Table 4: Evaluation of different media for the efficiency of shoot induction

Medium	% Response ±S.D	No. of shoot buds/ explant	No. of days for shoot induction
MS (Murashighe and Skoog)	93.17 ± 0.21	3 to 4	2 to 4
SH (Schienk and Hilderbrant,)	65.86 ± 1.12	1 to 2	5 to 7
WPM (Woody plant medium)	79.52 ± 0.67	1 to 2	3 to 6

Light influences the micropropagation through photoperiod, intensity and spectral quality (Athanasios *et al.*, 1987). The light intensity levels were studied at 1000 to 4000 lux. Of all the light treatments 2000 lux for 18 hr resulted in maximum shoot bud induction within a short time. While light below 2000 lux delayed it and resulted in yellowing of shoots. Light above 2000 lux resulted in rapid shoot bud initiation, but they failed to grow further.

The effect of three different media (MS, WPM and SH) on the shoot induction from nodal explants was studied (Table 4). These media differed mainly in their basal inorganic components such as nitrates and sulfates. The compared media were supplemented with the same amounts of sucrose, agar and phytohormones. MS medium was found to be superior since the number of days for shoot bud induction was lesser followed by WPM and SH (Rao and Farooq, 1994).

Carbohydrates act as a source of energy and as osmotic agents (Deburg, 1977). Sucrose is supplied as the main carbohydrate in the medium. Different concentrations of sucrose (0, 0.5, 1, 2, 3, 4 and 5%) were studied. Of which 2% sucrose was found to be optimum. Sucrose lower than 2% delayed shoot induction, whereas higher concentrations induced shoot buds, which failed to grow further. Similar findings were earlier reported in tobacco callus cultures by Brown *et al.* (1979).

Different phytohormones (BAP, Kinetin, NAA, IAA and IBA) at varying concentrations were used in the medium either singly or in combination with others and their effects on the shoot bud induction, growth of shoots and rhizogenesis was systematically evaluated (Table 3). Kinetin and BAP (0 to 4 mg/l) were used either singly or in combination with each other. The frequency of shoot induction with either Kinetin or BAP alone was very low and time for the initial leaf pulvinus abscission and shoot bud formation was 4 to 14 days. Usually one or occasionally two shoot buds formed in most of the nodal explants. Callusing was observed at higher concentrations of Kinetin and BAP (4 mg/l) along with a decrease in number of buds. When Cytokinin was eliminated there was no induction of buds in explants.

Double hormone treatments (Kinetin and BAP) in equimolar concentration had a profound influence on the initial leaf pulvinus abscission and the time taken for shoot bud induction. Similar observation was reported by Nair *et al.* (1984 b). The best response was observed with Kinetin 0.6 mg/l + BAP 0.6 mg/l, where as with Kinetin 0.8 mg/l + BAP 0.8 mg/l slight callusing was observed with decrease in number of buds. With BAP 1 mg/l + Kinetin l-4 mg/l, there was a gradual fall in number of induced buds per explant with increase in the callusing at cut ends. Polyphenol leaching from the explants was also prominent.

Triple hormone treatments included auxin (NAA) along with

BAP and Kinetin in equimolar concentrations. With Kinetin 0.6 mg/l + BAP 0.6 mg/l + NAA 0.2 mg/l, shoot bud induction and the number of buds per explant was reduced. However, when NAA was doubled to 0.4 mg/l keeping the other two constant, there was an increase in callusing with decreased shoot bud formation. Similarly, when NAA was increased to 0.6 mg/l there was further increase in callus.

The nodal explants along with induced buds were transferred to media containing MS basal + Kinetin 0.6 mg/l + BAP 0.6 mg/l + 3% sucrose + 0.8% agar and cultured for a month. To induce elongation the induced shoot buds were separated and cultured in MS basal + 0.2 mg/l Kinetin + 3% Sucrose + 0.1% Charcoal. In 20 to 25 days, these isolated buds have grown to a size of 2-3 cm and continued to grow further with occasional multiple branches.

In vitro grown shoots were subcultured to increase the number of propagules in the following ways I) MS basal liquid medium containing 0.1% Charcoal for 15 days. ii) Whites (1963) solid medium containing 1 mg/l BAP+ 2% Sucrose and iii) in Whites liquid medium supplemented with 1 mg/l BAP + 2% Sucrose. After 45 days, the explants cultured in Whites solid medium, produced 5 to 6 shoots per explant, where as explants cultured in Whites liquid medium suffered necrosis and failed to produce buds.

In vitro rhizogenesis in *Annona* was found to be very difficult. After multiplication, the shoots (2 to 4 cm) were used for rhizogenesis. Since the shoots taken from the multiplication medium failed to respond to rooting treatment, they were first cultured in MS basal medium containing 2% Sucrose + 1 gm/l activated Charcoal for 15 days and then were used for rhizogenesis. Nair *et al*. (1984 a) reported rooting of atemoya in MS semi-solid medium. In present study, White's media were used for root induction in both liquid and solid phases. Presence of 2% Sucrose and 0.1% activated Charcoal was found to be ideal for *in vitro* rooting.

IAA, IBA, NAA were used for inducing roots from the regenerated shoots at varying concentrations ranging from 0-100 mg/l. IAA and NAA were ineffective in inducing the roots and resulted in callus formation at the base of the shoot even at low concentrations, while, IBA was found to be effective in inducing roots. At lower concentrations of IBA (below 55 mg/l) swelling of the cut ends of the shoots was observed. In *Annona cherimola*, Encina *et al*. (1994) reported the use of high concentrations of IBA for rooting. In present study, *in vitro* grown shoots cultured in liquid White's medium for 3 days in dark and 7 days in light followed by a transfer to White's solid medium without hormones produced thin hairy roots. Best response was obtained when shoots were cultured in 100 mg/l IBA, but defoliation occurred. The rhizogenesis obtained in this study is an improved rooting response over the reports of Nair *et al*. (1984 a,b) in shoots derived from leaf culture and the endosperm culture (Nair *et al*., 1986). Roots were also induced with 110 mg/l IBA however, defoliation of shoots occurred within 2 days after root induction.

For acclimatization, rooted plantlets were first washed carefully with distilled water to remove agar sticking to the roots and the plantlets were carefully transferred to smaller plastic seed plugs containing a mixture of sterile vermiculate and sand (1:1) and covered to maintain abundant moisture around the plant and incubated at 24±2°C with 18 hours photoperiod. After one week, covers were removed and plants were exposed to external moisture levels gradually. Plants were transferred to bigger plastic pots containing the same mixture and placed in green house. Elongation was observed in transplanted shoots and new leaves emerged after 25 to 30 days. After two weeks, plants were transferred to earthen pots for further hardening.

References

Amin, M.N and V.S. Jaiswal, 1987. Rapid clonal propagation of Guava through *in vitro* shoot proliferation on nodal explants of mature trees. Plant Cell Tissue Organ Cult., 9: 235-243.

Athanasios, S., S. Economou and P.E. Read, 1987. Light treatments to improve the efficiency of *in vitro* propagation systems. Hort. Sci., 22: 751-754.

Brown, D.C.W., D.W.M. Leung and T. A. Thorpe, 1979. Osmotic requirement for shoot formation in Tobacco callus. Physiol. Pl., 46: 36-41.

Deburg, P., 1977. Symposium on tissue culture for horticultural purposes. Acta. Hort., 78: 459.

D'Silva, I. and D'Souza, 1993. Controlling contamination and browning of *in vitro* cultures of Cashew. J. Plantation Crops, 21: 22-29.

Encina, C.L., A. Barcelo-Munaz, A. Herrero-Castano and F. Pliego-Alfaro, 1994. *In vitro* morphogenesis of juvenile *Annona cherimola* Mill. bud explants. J. Hort. Sci., 69: 1053-1059.

Fridburg, G., N. Pederson, L. Landstram and J. Eriksson, 1978. The effect of activated charcoal on tissue culture; adsorption of metabolites inhibiting morphogenesis. Physiol. Pl., 43: 104-106.

George, A.P. and R.J. Nissen, 1987. Propagation of *Annona* species: A review. Hort. Sci., 33: 75-85.

Lemos, E.S.P. and J. Blake, 1996. Micropropagation of juvenile and adult *Annona squamosa*. Pl. Cell Tissue Organ. Cult., 46:77-79.

Lloyd, G. and B. McCrown, 1980. Commercially feasible micropropagation of mountain Laurel *Kalamia latifolia* by the use of shoot tip culture. Int. Plant Prop. Soc. Proc., 30: 421-427.

Murashige, T and F. Skoog, 1962. A revised medium for rapid growth and bioassays with Tobacco tissue culture. Physiol. Pl., 15: 473-497.

Nair, S., P.K. Gupta and A.F. Mascarenhas, 1984 a. *In vitro* propagation of *Annona* Hybrid. Indian J. Hort., 41: 160-165.

Nair, S., P.K. Gupta, M.V. Shirgurkar and A.F. Mascarenhas, 1984b. *In vitro* organogenesis from leaf explants of *Annona squamosa* Linn. Pl. Cell Tissue Org. Cult., 3: 29-40.

Nair, S., M.V. Shirgurkar and A.F. Mascarenhas, 1986. Studies on endosperm culture of *Annona squamosa* Linn. Plant Cell Rep., 5: 132-135.

Philips R., S.M. Arnot. and S.E. Kaplan, 1981. Antibiotics in plant tissue cultures: Rifamycin effectively controls bacterial contaminants without affecting the growth of short-term explant cultures of *Helianthus tuberosus*. Pl. Sci. Lett., 21: 235-240.

Pollock, K., D.G. Barfield and R. Shields, 1983. The toxicity of antibiotics to plant cell cultures. Plant Cell Rep., 2: 36-39.

Rao, T.V. and S.A. Farooq, 1994. Preliminary investigations on the *in vitro* studies in Annona squamosa. Front. Pl. Sci., 1: 261-264.

Rasai, S., A.P. George and A.S. Kantharajah, 1995. Tissue culture of *Annona* spp. (Cherimoya, atemoya, sugar apple and sour sop). A review. Sci. Horti., 62: 1-14.

Schenk, R.U. and A.C. Hilderbrandt, 1972. Medium and techniques for induction and growth of Monocot and Dicot plant cell cultures. Can. J. Bot., 50: 199-204.

Shields, R., J.S. Robinson and A.P. Anslow, 1984. Use of Fungicides in plant tissue cultures. Plant Cell Rep., 3: 33-36.

Tizzari, L., P. Pestelli, P. Fiorino and G. Parri, 1990. Propagation techniques for *Annona cherimola* Mill. Acta Hort. 275: 315-321.

Weatherhead, M.A., L. Burdon and G.G. Senshaw, 1978. Some effects of the activated charcoal as an additive to Plant Tissue Culture. II. Pflanzen. Physiol., 89: 141-147.

White, P.R., 1963. The cultivation of animal and plant cells. Ronald Press Co. New York, 228.

Zimmerman, H.R and F. Ingrid, 1989. Explant orientation affects axillary shoot proliferation of apple cultivars *in vitro*. Hort. Sci., 24: 351-352.